fatal arrhythmia and ventricular ischemia due to the restricted ventricles and enlarged atria. The transgenic mouse model provides us with a good tool to study the mechanisms and the cause of the death of RCM, which will be useful for the prevention and treatment of the disease.

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Both PKA Treatment and Cardiac Troponin-I N-Terminal Phosphorylation Alone Decrease Ca-Sensitivity and Eliminate Length-Dependent Activation in Skinned Cardiac Muscle

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Protein kinase A (PKA) phosphorylation of myofibrillar proteins constitutes an important pathway for β-adrenergic modulation of cardiac contractility. PKA targets the cardiac troponin I (cTnI) N-terminus, cardiac myosin-binding protein C (cMyBP-C) and titin. To isolate cTnI and cMyBP-C /titin phosphorylation effects on force-[Ca^{2+}] relations, endogenous cardiac troponin (Tn) was exchanged in rat, skinned trabeculae with either WT Tn or Tn containing a non-phosphorylatable mutant cTnI(S23/24A) or phosphomimetic cTnI(S23/24D). PKA cannot phosphorylate either cTnI mutant, leaving cMyBP-C and titin as sole PKA targets. Force-[Ca^{2+}] relations and Ca^{2+}-sensitivity (pCa50) were measured at 2.3 and 2.0 μm SL. Decreasing steady SL reduced maximal force (Fmax) and pCa50 similarly with WT Tn and Tn containing cTnI(S23/24A). PKA treatment of native, WT and cTnI(S23/24A) exchanged trabeculae reduced pCa50 at 2.3, but not 2.0 um SL, eliminating SL-dependence of pCa50. Reconstitution with Tn containing cTnI(S23/24D) reduced pCa50 at both SL (compared to WT and cTnI(S23,24A) and eliminated pCa50 SL-dependence; PKA did not significantly alter pCa50 at either SL. At each SL Fmax was similar with WT and mutant troponins, and was unaffected by PKA. Low angle x-ray diffraction experiments were performed to determine whether shifts in pCa50 were associated with changes in myofilament spacing (D1,0) or interaction. D1,0 at 2.3 um SL was similar in native trabeculae, with WT Tn and Tn containing either cTnI(S23,24A) or cTnI(S23,24D); PKA increased D1,0 in all cases. The results suggest that PKA phosphorylation of either cTnI or cMyBP-C /titin reduced the Ca^{2+}-sensitivity of force and length-dependent activation. Supported by NIH HL067071-06.

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Reducing Thin Filament Ca^{2+} Affinity with a cTnC Variant (L57Q) Reduces Force but Enhances Cross-Bridge Dependence of Cooperative Activation in Demembranated Rat Trabeculae

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Activation of cardiac contraction is initiated by binding of Ca2+ to troponin C (cTnC) and regulated by cooperative strong cross-bridge binding. We previously showed that passive exchange with cTn containing a cTnC variant, L48Q, increased Ca2+ sensitivity of force development and eliminated sarcomere length (SL) dependence of Ca2+ sensitivity of force in rat cardiac trabeculae. This was shown to be due to decreased reliance on strong cross-bridge binding for full thin-filament activation, possibly due to stronger cTnC-cTnI interaction. We also showed that PKA phosphorylation decreased Ca2+ sensitivity of force and eliminated SL dependence of force-pCa relations, by unknown specific mechanisms. Here we test the hypothesis that incorporation of a cTnC variant with decreased Ca2+ binding affinity, L57Q cTnC, will result in increased reliance on cross-bridge binding for full activation, thus increasing SL-dependence of Ca2+ sensitivity. As expected, results indicate trabeculae passively exchanged with L57Q cTnC-cTn displayed decreased Ca2+ sensitivity and rate of force production compared to WT cTnC at a given [Ca2+]. Interestingly, preliminary results indicate L57Q cTnC-cTn mildly increased the SL-dependence of Ca2+ sensitivity of force and also significantly decreased maximal force. Both of these effects were not observed in PKA-treated trabeculae, which had a comparable reduction in Ca2+ sensitivity of force. These findings suggest that reducing cTnC Ca2+ affinity per se can reduce Ca2+ sensitivity of contractile activation to the point of limited overall force production, which may enhance cross-bridge dependence of cooperative thin filament activation. Current experiments aim to increase force in L57Q cTnC (to near WT cTnC) by increasing cross-bridge attachment using 2'-deoxy-ATP, which has previously been shown to increase force and Ca2+ sensitivity while maintaining SL dependence. NIH-HL65497(MR), AHA-2310117(FSK).

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Fluorescence Measurements Using Rhodamine-Labeled cTnC Mutants Indicate Little Cooperative Interaction Between Cardiac Thin Filament Regulatory Units

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Exchange of mixtures of WT cTnC and mutant cTnC(D65A), which cannot bind Ca^{2+} at N-terminal site II (''dead'' cTnC), reduced maximal Ca^{2+} activated force (Fmax) with little effect on force-Ca^{2+} relations and force kinetics in skinned cardiac trabeculae (Gillis et al., J Physiol. 580:561-76, 2007), suggesting interaction between structural regulatory units (RUs; 7 actins, 1 troponin, 1 tropomyosin) along cardiac thin filaments is less than in skeletal muscle (Regnier et al., J Physiol. 540:485-97, 2002). To more directly test that this finding, we exchanged skinned cardiac trabeculae with mixtures of mutant cTnC(C35S) and cTnC(C35S,D65A), with one or the other labeled at Cys 84 with 5'tetramethyl rhodamine (IATR) for dichroism measurements. In trabeculae exchanged with 100% cTnC(C35S)-IATR, dichroism increased in response to both Ca^{2+} and rigor crossbridges, while trabeculae with 100% Tn containing (cTnC(C35S,D65A)-IATR) had no response to Ca^{2+}, but retained a strong response to rigor crossbridge binding. This response to strong crossbridges allows use of cTnC(C35S,D65A)-IATR to determine if isolated regulatory units containing cTnC(C35S,D65A)-IATR are perturbed by Ca^{2+}-induced active contraction in adjacent ''live'' RUs. To test this, trabeculae were exchanged with a mixture of 20% functional cTnC(C35S)-IATR and 80% unlabeled cTnC(C35S,D65A), to isolate functional RUs. Fmax decreased but there was little change in the Ca^{2+}-dependence of dichroism compared to trabeculae exchanged with 100% functional cTnC(C35S)-IATR. These data indicate minimal or no apparent spread of activation between adjacent RUs in cardiac muscle, indicating that the apparent cooperativity of force production in cardiac muscle results from interactions between myosin and thin filaments within a thin filament structural regulatory unit. Supported by NIH RO1-HL65497 (Regnier).

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Combination of Phosphomimetic Substitutions within Cardiac Troponin I Cause Functional Cross-Talk

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Protein kinase C (PKC) phosphorylates 3 clusters of residues within cardiac troponin I (cTnI) and yet, it is unclear whether phosphorylation at multiple sites produces additive and/or divergent functional modifications. Our goal was to evaluate the influence of cTnI with phosphomimetic substitutions on contractile performance under basal conditions and in response to PKC activation by endothelin. Endogenous cTnI was replaced with phosphomimetic substitutions using adenoviral-mediated gene transfer into adult rat cardiac myocytes. Phosphomimetics of Ser43/45, Ser43/45 plus Thr144, and Ser23/24 plus Ser43/45 were substituted with Asp to form AdcTnISer43/45Asp, $cTnIAsp_{Triple}$ (e.g. cTnISer43/45AspThrT144Asp), and $cTnIAsp_{Quad}$ (cTnISer23/24/43/45Asp). Isolated myocytes were electronically paced and studied 4 days after gene transfer. Gene transfer of epitope-tagged versions of each construct resulted in 30-40% replacement after 2 days and >65% replacement of endogenous cTnI 4 days after gene transfer without significant alterations in the expression of other myofilament proteins. In functional studies, peak shortening amplitude was significantly decreased in myocytes expressing cTnISer43/45Asp or $cTnIAsp_{Quad}$, while peak shortening in myocytes expressing $cTnIAsp_{Triple}$ was not significantly different from controls. Relaxation was accelerated in myocytes expressing $cTnIAsp_{Quad}$, but was not different from controls in myocytes expressing $cTnIAsp_{Triple}$ or cTnISer43/45Asp. Together, these results suggest the Ser23/24 and Ser43/45 sites have an additive influence on shortening, while substitution at Thr144 attenuates the influence of Ser43/45 on peak shortening. To further determine whether multiple phosphomimetic substitutions within cTnI influence myocyte shortening, we studied the change in peak shortening and relaxation produced by the PKC agonist, endothelin (10 nM). In preliminary studies, the increased peak amplitude and accelerated relaxation observed in control myocytes is not significantly different in myocytes expressing cTnISer43/45Asp. However, there is a trend for myocytes expressing $cTnIAsp_{Quad}$ to show an attenuated amplitude and relaxation response to ET.

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The Motif of Myosin Binding Protein-C is Mechanically Weak and Extensible

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Cardiac myosin binding protein-C (cMyBP-C) is a member of the immunoglobulin (Ig) superfamily of proteins and consists of 8 Ig- and 3 fibronectin (Fn)-like domains along with a unique regulatory sequence referred to as the MyBP-C ''motif'' or M-domain. The structure of the M-domain is not known, but small angle X-ray scattering experiments suggest that it adopts a compact shape in solution and that its overall dimensions are similar to other Ig-like domains. To investigate whether the M-domain behaves similarly to an Ig domain under mechanical stress or load, we used atomic force microscopy (AFM) to investigate single molecule elasticity and mechanical properties of recombinant full-length mouse cardiac cMyBP-C and smaller proteins containing just the M-domain and flanking Ig- sequences. Force-extension curves of full-length cMyBP-C showed unfolding of individual Ig or Fn-like domains at forces